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<b>(54) Title:</b> GROWTH HORMONE RELATED PEPTIDE  <b>(57) Abstract</b>  Antigenic molecules cause antibodies to be raised against at least some of the 112 to 159 region of a natural growth hormone when injected <i>in vivo</i> . The molecules can comprise portions of the 112 to 159 region. The antibodies are believed to bind to growth hormone on administration and enhance its effect.		

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GROWTH HORMONE RELATED PEPTIDE  
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The present invention relates to biologically active molecules, particularly peptides, more particularly peptide fragments of growth hormone (including bovine growth hormone (bGH), porcine growth hormone (pGH), chicken growth hormone (cGH), ovine growth hormone (oGH) and their mutant derivatives), which can enhance or promote growth hormone activity. Human growth hormone (hGH), rat growth hormone (rGH), mouse growth hormone (mGH), horse growth hormone (eGH) and salmon growth hormone (sGH) are other growth hormones of interest.

Polypeptide hormones are important for both medical and veterinary application. One such hormone, growth hormone, is found in vertebrates and is important for promoting somatic growth. Growth hormones from different species share both structural and functional characteristics. Growth hormones consist of amino acid sequences generally of about 191 residues in length. It is known that growth hormone can stimulate somatic growth, promote wool growth in sheep, affect body composition, improve food efficiency and promote lactation in appropriate species. Different aspects of the structural and functional characteristics of growth hormones have been described (Nicoll et al, 1986; Isaksson et al 1985; Wallis, 1978).

Antibodies to hormones have been shown to be capable of (i) enhancing hormone activity, (ii) have no effect on hormone activity or (iii) inhibit hormone activity (Thompson 1937; Rolands 1939; Goodfriend et al, 1970;

Shechter 1979a,1979b ; Cole et al 1975; Aston et al, 1986,1987 Ferguson 1954). More specifically, Aston et al (1986,1987) have shown that certain antibodies to growth hormones can enhance the biological activity of the hormone in vivo. It was concluded in these studies that enhancement of hormone by antibodies is characteristic of particular specificities, a property also described previously (Cole et al 1975; Goodfriend et al 1970). However, in none of these studies is a regime available to indicate how consistently to produce an enhancing antiserum by using a small peptide. EP-A-0137234 discloses that the large 7 kiloDalton fragment of growth hormone can produce antibodies that enhance growth hormone activity; however, this fragment may not always be suited for this purpose due to its size and its limited ability to produce a consistent enhancing antiserum. Currently, manufacture of such a large polypeptide may be problematic by peptide synthetic routes.

Enhancement of hormonal activity by the administration of a peptide fragment of GH has been disclosed in WO-A-8404915). In this particular disclosure it was shown that a short peptide derived from the amino terminal portion of the GH molecule potentiated hypoglycaemic activity; however this peptide was not administered in an immunogenic fashion. Both GH and insulin induce hypoglycaemia when administered to animals. The insulin-potentiating activity of peptide fragments of GH have also been described elsewhere (Pullin et al 1981; Ng et al 1980).

This invention relates to various specific peptides and

their derivatives which have the capability of inducing antibodies to enhance growth hormone activity. At least some of the peptides may have growth hormone activity themselves.

According to a first aspect of the invention, there is provided an antigenic molecule which causes antibodies to be raised against at least some of the 112 to 159 region of a natural growth hormone. This may be achieved by providing a molecule having antigenic equivalence to at least part of the 112 to 159 region.

According to a second aspect of the invention, there is provided a molecule (other than a natural growth hormone) at least part of which is antigenically equivalent to an oligopeptide selected from residues 112 to 159 of a natural growth hormone.

The 112 to 159 regions of various natural growth hormones have the following amino acid sequences:

	112	120	130	140	150	159
bGH						
hGH	KLKDL	EEGIL	ALMRE	LEDGTP	RAGQIL	KQTYDK
pGH	KLKDL	EEGIQ	ALMRE	LEDGSP	RAGQIL	KQTYDK
oGH	KLKDL	EEGIL	ALMRE	LEDVTP	RAGQIL	KQTYDK
eGH	KLRDL	EEGIQ	ALMRE	LEDGSP	RAGQIL	KQTYDK
mGH	LLKDL	EEGIQ	TLMGR	LEDGSS	RGTGQI	FKQTY
cGH	KLKDL	EEGIQ	ALMRE	LEDGSP	RGPQLL	RPTYDK
rGH	KLKDL	EEGIQ	ALMQE	LEDGSP	RIGQIL	KQTYDK
sGH	VGINL	LITGS	QDGVLS	LDDNDS	QQLPY	GNYYQN
						LG
						GD
						GN
						VR
						RR
						NY
						EL

Bovine, ovine, porcine and chicken growth hormones are

preferred. Ovine growth hormone is very similar to bGH.

In the above, and throughout this specification, the amino acid residues are designated by the usual IUPAC single letter nomenclature. The more recent single letter designations may be correlated with the classical three letter designations of amino acid residues as follows:

A = Ala	G = Gly	M = Met	S = Ser
C = Cys	H = His	N = Asn	T = Thr
D = Asp	I = Ile	P = Pro	V = Val
E = Glu	K = Lys	Q = Gln	W = Trp
F = Phe	L = Leu	R = Arg	Y = Tyr

The oligopeptide will be of at least the minimum size necessary to confer antigenicity: usually it will be of at least six or seven residues, but may be of any suitable length up to, for example, 20 amino acid residues. The best oligopeptides may be expected to correspond to topographical surface features of a natural growth hormone molecule, that is to say those features having some three-dimensional feature protruding from or extending into the ambient surface level of the hormone. Preferred oligopeptides correspond to regions 133-159 and 128-147.

Probably the most simple way of ensuring that at least part of the molecule is antigenically equivalent to the oligopeptide is for that part of the molecule to comprise a sequence of amino acid residues which is identical to or conformationally similar to the oligopeptide. However, any other way of producing

antigenic equivalence may be used: an example is to use an anti-idiotypic antibody or other (even non-proteinaceous) analogue.

The invention therefore encompasses short peptides (preferably of less than 20 amino acid residues, but generally of at least six or seven amino acid residues, for example eight to twelve residues) sharing structural homology with growth hormone and which when administered to an animal can enhance hormone activity. Potentiation of hormone activity (in particular growth hormone activity), may occur through direct or indirect effects on the hormone in question.

The invention therefore encompasses a peptide other than a natural growth hormone comprising the amino acid sequence:

GTPRAGQILKQTYDKFDTNMR

or an active fragment thereof and/or conservative mutant thereof. This sequence is taken from bGH, peptides 130 to 150.

The peptide will generally be antigenic and capable of stimulating the production of antibodies which, when in an appropriate formulation, potentiate the effect of growth hormone.

As stated above, an active subfragment of the specified sequence may be used. Active subfragments may consist of or include hexapeptides, including one or more of:



GTPRAG	QILKQT	YDKFDT
TPRAGQ	ILKQTY	DKFDTN
PRAGQI	LKQTYD	KFDTNM
RAGQIL	KQTYDK	FDTNMR
AGQILK	QTYDKF	
GQILKQ	TYDKFD	

Active subfragments may also consist of or include heptapeptides, including one or more of:

GTPRAGQ	ILKQTYD	KFDTNMR
TPRAGQI	LKQTYDK	
PRAGQIL	KQTYDKF	
RAGQILK	QTYDKFD	
AGQILKQ	TYDKFDT	
GQILKQT	YDKFDTN	
QILKQTY	DKFDTNM	

The invention also encompasses a peptide other than a natural growth hormone comprising the amino acid sequence:

AGQILKQTYDKFDTNLRSDDA

or an active fragment thereof and/or conservative mutant thereof. The above sequence is taken from pGH, peptides 134 to 154.

Active subfragments may consist of or include hexapeptides, including one or more of:

AGQILK	QTYDKF	DTNLRS
GQILKQ	TYDKFD	TNLRSD



QILKQT	YDKFDT	NLRSDD
ILKQTY	DKFDTN	LRSDDA
LKQTYD	KFDTNL	
KQTYDK	FDTNLR	

Active subfragments may also consist of or include heptapeptides, including one or more of:

AGQILKQ	TYDKFDT	NLRSDDA
GQILKQT	YDKFDTN	
QILKQTY	DKFDTNL	
ILKQTYD	KFDTNLR	
LKQTYDK	FDTNLRS	
KQTYDKF	DTNLRSD	
QTYDKFD	TNLRSD	

The invention further encompasses a peptide other than a natural growth hormone comprising the amino acid sequence:

IQALMRELEDGSPRAGQILKQ

or an active fragment thereof and/or conservative mutant thereof or salts thereof. This sequence is taken from pGH, peptides 120 to 140.

Active subfragments may consist of or include hexapeptides, including one or more of:

IQALMR	ELEDGS	PRAGQI
QALMRE	LEDGSP	RAGQIL
ALMREL	EDGSPR	AGQILK
LMRELE	DGSPRA	GQILKQ

MRELED	GSPRAG
RELEDG	SPRAGQ

Active subfragments may also consist of or include heptapeptides, including one or more of:

IQALMRE	LEDGSPR	AGQILKQ
QALMREL	EDGSPRA	
ALMRELE	DGSPRAG	
LMRELED	GSPRAGQ	
MRELEDG	SPRAGQI	
RELEDGS	PRAGQIL	
ELEDGSP	RAGQILK	

Other preferred sequences include:

EDGSPRAGQIL  
AGQILKQTYDK  
RAGQILKQTYDKFDTNLRSD  
DTNLRSDALL  
KQTYDKFDTNLRSDALLKNY  
LRSDALLKNY

and active fragments (including the hexa- and heptapeptides) thereof and/or conservative mutants thereof or salts thereof.

It should be noted that combinations of more than one of the above sequences may be used.

According to a further aspect of the invention, a method of promoting the activity of growth hormone (or a substance having growth hormone activity) comprises

administering to a vertebrate an effective amount of a peptide or other molecule as described above. The invention thus encompasses the use of a peptide or other molecule as described above in the preparation of an agent for use in the promotion of the activity of growth hormone or a substance having growth hormone activity.

Peptides and other molecules in accordance with the invention may be presented in a variety of ways. For preference, an antigenic region (such as a peptide fragment or sub-fragment) in a molecule in accordance with the invention will contain the amino acid sequence of choice linked to a carrier peptide or protein. It is generally preferred to have a plurality, for example 5 to 10, copies of a peptide sequence (for example one or more of the above sequences) linked to the carrier. The carrier can for convenience be a generally large protein, which is inert in material respects, and which is derived from a different species or genus from that associated with the natural growth hormone. Examples of carriers include albumins such as human serum albumin, bovine serum albumin and ovalbumin (although not so many peptides will probably be able to be carried in this last case). Alternatively, keyhole limpet haemocyanin can be used. The carrier will generally preferably come from a different species from that on which the fragment is based.

It is not essential that peptide sequences as described above be linked to albumins: they may be linked to other macromolecules, such as beta-galactosidase, especially of bacterial origin.

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The invention encompasses molecules being peptides or having peptide regions which share substantial (eg greater than 30%, 50% or even 70%) sequence homology) with the above peptides. Similarly, conservative amino acid substitutions may not decrease the immunogenicity or antigenicity of peptides. Thus antigenically similar homologues will elicit antibody which binds to GHs in the same region as the above peptides define. It is well known that the use of homologues can be a means of circumventing 'self' tolerance. Thus the use of the corresponding sequences from other species may be advantageous in this invention. Examples of homologous sequences to the preferred bovine, porcine and ovine growth hormones are those derived from the corresponding sequence regions of equine GH, mouse GH, chicken GH, trout or salmon GH or rat GH.

It is alternatively possible for molecules in accordance with the invention which are or which comprise peptides to be or to include polymers of sequences as described above. Appropriate sequences can be polymerised either by cross-linking of two cysteine residues to form disulphide bonds or by using external chemical coupling agents (such as carbodiimide, glutaraldehyde or other dialdehydes or di- (or poly-) functional carboxylic acids. As a further alternative, recombinant DNA techniques could be used to produce a peptide polymer.

It should be noted that the chemical coupling (which could for example take place through the agency of lysine residues) and disulphide bond formation are not

limited to when the coupling residues are at the end of the sequence: internal residues could also be appropriate. Coupling residues, for example cysteine residues, may be added as desired.

It may be found that it is not necessary to couple any of the sequences described above with external peptides. They may be antigenic on their own. In such a case, it may be advisable to select particular adjuvants such as DEAE dextran and Merck 7426.

According to a further aspect of the present invention, there is provided a pharmaceutical or veterinary composition comprising a molecule as described above in conjunction with a pharmaceutically or veterinarily acceptable carrier. The composition may contain an adjuvant, for example, DEAE dextran, Merck 7426, saponin and aluminium hydrogel. Alternatively or in addition Freund's complete adjuvant could be used. As noted above, certain adjuvants are more likely to be preferred in particular circumstances.

Compositions in accordance with the invention will normally be sterile, as they will be intended for implantation or injection. Intravenous injection is not preferred: subcutaneous injection is the route of choice, although possibly intramuscular and/or intraperitoneal injection could be used.

The preferred location of the subcutaneous injection would be at the back of the neck in the case of an animal, as that is a place where commercially useful meat is less likely to be damaged by tissue breakage or

bruising.

The carrier will generally be an isotonic buffer plus saline, such as PBS or physiological saline.

Dosages will be generally as prescribed in accordance with the directions of the physician or veterinary surgeon, but 5 to 500 mcg per dose, particularly 50 to 100 mcg of peptide or other molecule may be found to be suitable for bovine or porcine applications.

A substance having or promoting growth hormone activity can be administered shortly after (or may be in some circumstances prior to or at the same time as) peptides or other molecules in accordance with the present invention. The activity of the growth hormone is then enhanced. This can lead to enhanced growth where growth is relevant; improved body compositions (for example, in pigs there may be less fat and more muscle on the back); better wool growth in sheep; improved growth efficiency (that is to say, better growth for a given unit amount fed); and enhancement of lactation in cows and sheep. This latter application is not only important for providing milk for human consumption, but it may also enable sheep to rear their young more effectively.

Examples of substances promoting growth hormone activity other than growth hormones themselves and other than molecules in accordance with this invention include antibodies to growth hormone inhibitors and antibodies to other growth hormone antagonists, such as antibodies against somatostatin or leutinising hormone

releasing hormone (LHRH). The production of an antibody to somatostatin, for example, would increase circulating GH levels and may thus potentiate the effect of molecules in accordance with the present invention. Another substance which may be regarded as promoting growth hormone activity is growth hormone releasing hormone (GRF), which may also be administered.

In another aspect, the invention provides antibodies raised against molecules of the first aspect. Such antibodies may be parenterally administered to animals, generally in an appropriate formulation to produce a growth hormone potentiating effect. Preferred formulation and administration details may be as described above, with changes as appropriate.

The invention will now be illustrated by the following examples. The examples refer to the accompanying drawings, in which:

Figure 1 show regions of high antigenicity in pGH and bGH;

Figures 2, 3 and 4 show that molecules in accordance with the invention enhance dwarf mice growth rates; and

Figures 5a to 5e show the effect of antipeptide antiserum on sheep growth and metabolism.



Example 1 Synthesis of bGH 130-150 peptide containing C-terminal Cys-Ala - Sequence 1

Synthesis of peptides was primarily performed by the Fmoc-methodology, essentially as described by Dryland and Sheppard (1986). The peptide was assembled with the C-terminus covalently attached to a polymeric support consisting of polymerised polydimethylacrylamide (Atherton and Sheppard 1985). This material is commercially available as Pepsyn KA resin for the purpose of peptide synthesis.

Attachment of the first amino acid to the resin via an ester bond is effected using dimethylaminopyridine as the catalyst. Throughout the synthesis dimethylformamide was used as the solvent. Temporary Na-amino group protection is the base-labile 9-fluorenylmethoxycarbonyl (Fmoc) group, which is removed with 20% piperidine in DMF. Subsequent amino acids are added sequentially by a repetitive protocol of amino deprotection, followed by coupling of an activated amino acid.

The hydroxy side-chains of serine, threonine and tyrosine are protected as t-butyl ethers, the carboxyl side chains of aspartic and glutamic acids as their t-butyl esters. The basic side chains of lysine and histidine are protected with the t-Boc group, but the guanidino side-chain functionality of arginine is protected as the 4 methoxy 2,3,6-trimethylphenylsulphonyl derivative (Atherton et al 1983). Cysteine is protected as the S-acetamido methyl (ACM) or trityl derivative.

Amino acid derivatives were added as their pentafluorophenyl esters (PFP) with hydroxybenzotriazole (HOBT) as the catalyst, but serine and threonine are added as the 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (DHBT) esters (Atherton et al 1986). Occasionally, Fmoc amino acids were added as their pre-formed symmetrical anhydrides.

Acylation and deprotection steps were monitored spectrophotometrically as well as by the trinitrobenzene sulphonic acid (TNBSA) (Hancock and Battersby, 1976) test procedure and occasionally by amino acid analysis of the acid hydrolysate of resin bound peptide.

After the assembly of the peptide was complete, the peptide-resin bond was cleaved and most side chain protecting groups are removed simultaneously by the action of 95% trifluoroacetic acid (TFA)/scavenger mixture. Commonly used scavengers are water, phenol, ethanedithiol, ethylmethanethiol, anisole and thioanisole.

The choice of scavengers and condition for cleavage varied with the composition of the peptide. TFA is removed in vacuo and scavengers extracted into ether. The crude peptide can be recovered from the aqueous layer by lyophilisation.

Peptides were synthesised by the t-Boc/benzyl strategy. The support was either 4-(oxymethyl)-phenylacetamidomethyl (PAM), or benzhydrylamine (MBH) resin. Temporary

Na-protection is the acid labile t-butyloxycarbonyl (BOC) group, which is removed by trifluoroacetic acid (TFA) in dichloromethane. After neutralisation, the next Boc amino acid is added as the preformed symmetrical anhydride as, for example, programmed by Applied biosystems 430A peptide synthesiser. The assembled peptide was cleaved from the resin with anhydrous hydrogen fluoride (HF) which also removes the usually benzyl-based side chain protecting groups.

Purification of peptides was achieved by gel filtration, ion exchange chromatography and high performance liquid chromatography (HPLC) or a combination of these methods.

Homogeneity of peptides was assessed by analytical HPLC, thin layer chromatography (TLC) and electrophoresis on thin layers of cellulose. Amino acid analysis of the acid hydrolysate and determination of the amino acid sequence of the synthetic peptide were also employed to indicate purity of the final product.

Peptides were optionally prepared with a Cys or Cys-Ala residue(s) at the carboxy-terminal for ease of synthesis or for alternative cross linking procedures.

Example 2 Synthesis of pGH 134-154 peptide containing C-terminal Cys-Ala - Sequence 2

Following the methodology of Example 1, the above sequence was synthesised.

Example 3 Synthesis of bGH 120-140 peptide containing C-terminal Cys-Ala - Sequence 3

Following the methodology of Example 1, the above sequence was synthesised.

Examples 4 to 6 Preparation of carrier-linked immunogenic vaccines

Example 4

A synthetic peptide as prepared in Example 1 (Sequence 1) was cross-linked to 'carrier' (ovalbumin) by using glutaraldehyde. The immunogen consisted of 5 mg peptide mixed with an equal weight of 'carrier' (ovalbumin, 5 mg), dissolved in a total volume of 4ml phosphate-buffered saline (PBS) (25 mM sodium dihydrogen orthophosphate and 25mM disodium hydrogen orthophosphate containing 0.8% NaCl, pH 7.4). To this mixture glutaraldehyde (Sigma) solution was added. Generally the amount of cross-linking agent employed was 0.1 or 0.5 ml of a 0.5% solution (w/v) in water. The conjugation mixture was allowed to incubate at 20°C for between 30-60 minutes prior to dialysis and emulsification with adjuvant. It was found that dialysis was not a necessary step in the vaccine preparation and was sometimes omitted from the protocol.

Example 5

The procedure of Example 4 was followed, except that a

synthetic peptide as prepared in Example 2 (Sequence 2) was used.

#### Example 6

The procedure of Example 4 was followed, except that a synthetic peptide as prepared in Example 3 (sequence 3) was used.

#### Examples 7 to 9 Preparation of carrier-free immunogenic vaccines

Preparation of 'carrier free' immunogen was achieved by repeating the general procedures of Examples 4 to 6 but omitting the ovalbumin from the conjugation mixture. The vaccine of Example 7 is based on Sequence 1, that of Example 8 is based on Sequence 2 and that of Example 9 on Sequence 3.

#### Examples 10 to 12 Vaccination protocols in sheep

Peptide vaccines as prepared in each of Examples 4 to 6 in a total volume of 4ml of PBS were emulsified with 7ml of Freund's complete adjuvant and injected subcutaneously (SC) at two distinct sites in sheep (Merino). A second challenge (SC, two further sites) with the same vaccine was performed after 28 days in Freund's incomplete adjuvant and blood samples (jugular venepuncture) taken after 14 days and at weekly intervals thereafter. Further challenges with peptide vaccines were performed at 3-4 week intervals. Serum was prepared by allowing clotting of the blood whereas plasma was obtained by taking blood into heparinised

tubes. Freund's adjuvants were purchased from Difco Laboratories.

Vaccination of cattle and pigs may be performed under similar conditions.

Examples 13 to 15 Preparation of serum or plasma samples and assay of anti-growth hormone antibodies in serum or plasma samples

Assays for antibody were performed with diluted serum or plasma whereas growth experiments were conducted with the immunoglobulin fraction of serum prepared by sodium sulphate precipitation. Sera were precipitated with two volumes of sodium sulphate (27% w/v in water) and collected by centrifugation. Subsequently, the globulin fraction was dialysed extensively against PBS (Johnstone and Thorpe, 1982).

The immunogenicity and antigenicity of vaccines was determined by employing liquid-phase radioimmunoassay as described previously (Aston et al 1985). Essentially, serum or plasma was diluted in PBS (1/50-1/5000) and 0.05ml was assayed by the addition of 0.05ml of  $^{125}\text{I}$  labelled bGH or pGH. Binding of antibody in the serum to the tracer was determined by precipitation with a second antibody coupled to cellulose (SAC-CEL, Wellcome Diagnostics). The word SAC-CEL is a trade mark.

Solid phase assays (ELISA) were performed as follows. bGH and pGH were coated onto solid phase microtitre plates as described previously (Aston et al 1985)



except that the plates were blocked with 1% bovine serum albumin BSA instead of haemoglobin. Sera were diluted across the plates (1/50, 1/500, 1/5000, 1/50000) in 1% BSA in PBS and incubated at 37°C for 2 hours or overnight at 4°C. Binding of anti growth hormone antibody in sera to the plates was determined by the addition of rabbit anti sheep immunoglobulin conjugate (peroxidase) (Dakko, 1/1000 in 1% BSA/PBS). which was incubated at 0.1ml per well for 2 hours at 37°C. The substrate, 2,2-azino-di(3-ethyl benz-thiazolin-sulphonate(6)) (ABTS), (Boehringer Mannheim) was at a concentration of 1mg/ml in citrate buffer pH 4.2 and contained 1mcl of 30% hydrogen peroxide per 5ml. This was incubated at room temperature for five minutes before being read in a Dynatech MR600 microplate reader at 410nm.

#### Assay controls

Control sera or plasma samples were derived from sheep which had received 'carrier' protein cross-linked with glutaraldehyde in the absence of peptide. The control vaccines were administered to groups of animals in parallel to the peptide vaccine treatments. Sheep group sizes were generally 5-10 animals but on occasions were as high as 50 animals per group.

The results are shown in Tables 1 and 2, which indicate the generation of growth hormone cross reactive antibodies by immunisation with peptides.

It is shown that immunisation of sheep with short peptides within the region 112-159 can elicit



antibodies which bind to bGH and pGH (it is noteworthy that bGH and oGH differ in only one or two amino acids out of 191 and thus both hormones bind to the same antisera equally well). The efficacy of antibody production varies with the sequence being used. It is also shown that certain sequences give a better response if 'carrier' is used whereas others this aspect is of lower importance. The extensive sequence homology between growth hormones from different species may enable use of a bGH sequence to elicit high affinity antibody against pGH and vice versa. It is clearly indicated in Table 1 that bGH sequences elicit antibody recognising pGH. The use of homologues rather than a 'self' component to elicit auto antibody has been known for some time.

Table 1 shows the antibody response of sheep to peptide sequences from bovine and porcine GHs. Antibody was determined by liquid-phase radioimmunoassay (RIA). (Positives retained counts of 200-2000cpm above the serum of control immunized sheep).

Table 1

Sequence (1) bGH 130-150 )	Contain C-terminal Cys-Ala
Sequence (2) pGH 134-154 )	
Sequence (3) bGH 120-140 )	

% responding animals (n=5)

	bGH	pGH
Sequence (1)	60	80
Sequence (2)	70	40
Sequence (3)	80	80

The efficacy of the response did not depend on whether a carrier was employed. The same results were obtained whether the peptides were cross-linked to an ovalbumin carrier or polymerised to themselves.

Table 2 shows another sheep experiment on the antigenicity of sequences pGH 134-154 and bGH 120-140. n=50/group. Serum assayed corresponded to that taken 2 weeks after the second injection with peptide vaccine. In this case, the peptides were polymerised to themselves with glutaraldehyde in accordance with Examples 8 and 9.

Table 2

	% responding sheep	
	bGH	pGH
Sequence (2) pGH 134-154	54	56
Sequence (3) bGH 120-140	60	74

(Binding to antisera ranged from between 400-2000 cpm above that of control serum)

Examples 16 to 18 Determination of growth enhancing antisera in dwarf mice

Example 16

Metabolic assay of growth in Snell dwarf mice was performed as described previously (Aston et al, 1986, 1987; Holder et al, 1980). Essentially, male and female animals were randomised and allocated to cages so that no two mice of the same treatment group were in the same cage. Hormone or complex (prepared one hour prior to injection) with antipeptide antibodies were administered subcutaneously (0.1ml) on two consecutive days before injection of  $^{35}\text{S}$  labelled sodium sulphate (0.5 mCi/g) (Amersham Radiochemicals) for a further 24 hours. Incorporation of radioactivity was measured in costal cartilage which articulated directly with the sternum after removal of the soft tissue. The mean growth rate of each treatment group was expressed as dpm/mg cartilage and represents the uptake of  $^{35}\text{S}$ -sulphate from six animals. Significance was determined by Student's T-test. The direct relationship between somatic growth and incorporation of radioactivity into cartilage has been described previously (Aston et al 1986, 1987; Holder et al, 1985). The results are shown in Figure 2.

Figure 2 clearly shows enhancement of dwarf mouse growth rate by anti GH antisera, as determined by  $^{35}\text{S}$

sulphate incorporation into cartilage (Aston et al 1986,1987). Site directed antisera to bGH and pGH, raised by vaccinating sheep with peptide pGH 134-154 were examined for their effects in dwarf mice following complexing with pGH (50 mcg) prior to injection. Control animals received antibodies from sheep which had received a control vaccine (FCA/ovalbumin only). Each treatment group consisted of 6 animals. The three immune globulin preparations tested (A,B,C) were derived from different animals. Significance was determined by unpaired Student's T- test.

#### Example 17

In a second dwarf mouse experiment the growth enhancing activities of two anti-peptide antisera were examined. The results are shown in Figure 3. Mice were treated with bGH (50 mcg) mixed with either control immune globulin (first column) or with immune globulin derived from sheep vaccinated with peptides pGH 134-154 (second column), bGH 120-140 (third column) or a monoclonal antibody which enhances bGH activity (positive control; fourth column). Significant enhancement of the growth promoting activity of bGH was achieved with immunoglobulins raised with the above peptides.

#### Example 18

In a third dwarf mouse experiment further antisera raised to pGH 134-154 and bGH 120-140 were examined. The results are shown in Figure 4. Mice were treated with pGH (50 mcg) either mixed with control globulin or

immune globulin to pGH raised by vaccinating sheep with sequences pGH 134-154 or bGH 120-140. Growth rate was determined by incorporation of  $^{35}\text{S}$ -sulphate uptake into costal cartilage (Aston et al, 1986, 1987). Levels correspond to means  $\pm$  SEM. Weight gain was determined at the end of the experiment (hatched bars) and in all cases of treatment with pGH (50 mcg) + specific immune globulin (anti pGH 134-154 or bGH 120-140) significant increases in growth were observed ( $P < .001$ ).

Example 19 Effects of passive administration of anti-peptide immunoglobulin on sheep growth and metabolism

Sheep (pure Poll Dorset) were randomly allocated to one of four treatment groups ( $n=7$ ). The four groups were subjected to a pre-treatment period of 3 weeks and weighed twice per week. During the pre-treatment period blood was also taken at the times of weighing. On day 15, blood samples were taken hourly for 18 hours from a catheter. On day 18 an insulin tolerance test was performed on the animals. All animals were maintained on an ad lib diet of 16% crude protein farm mix (daily refusals were recorded).

Treatments were bGH, bGH+anti-peptide immunoglobulin, anti-peptide immunoglobulin only, and control globulin.

During the treatment period (18 days) the animals were maintained on the same diet as above and weighed twice/week. On day 8 of treatment, hourly blood samples were taken (18) whereas on day 10, another insulin tolerance test was performed (ITT). Eight animals were slaughtered on days 14, 15, 17 and the remaining four

on day 18. At slaughter, liver was weighed and samples of subcutaneous fat were taken. The fat samples were analysed for lipolytic and lipogenic activity and also for lipid oxidation. Lipogenesis was determined by  $^{14}\text{C}$  acetate incorporation into lipid, lipolysis by glycerol release (using BCL test kit 148270) and lipid oxidation by  $\text{CO}_2$  production as described previously (Smith and Walsh, 1984, 1988; Yang and Baldwin 1973)

The results are shown in Figures 5a to 5e. These show the effects of antipeptide antiserum (pGH 134-154) on sheep growth and metabolism. Immune globulin cross-reactive with oGH (also with bGH and pGH), raised in sheep by vaccination with peptide pGH 134-154, was administered to growing lambs (Dorset). The growth rate, liver weight, and metabolic activity of fat tissue was determined at slaughter. Treatments included (A) control immune globulin (from ovalbumin immunised sheep), (B) anti ovine GH antiserum raised by immunising sheep with pGH 134-154, (C) bovine GH (10mg), and combination of (B) + (C). Treatments were administered on alternate days.  $n=7$  animals/group.

Treatment of lambs with anti-pGH 134-154 immune globulin increased growth rate (B) although to a lesser degree than did growth hormone (C); liver weights were similarly increased.

Growth hormone is known to be diabetogenic when administered to animals, thus agents that enhance endogenous GH activity would be expected to manifest diabetogenic activity (resistance to insulin). Treatment of lambs with bGH or bGH in complex with



anti-pGH 134-154 antiserum results in significant diabetogenic activity. Administration of antiserum on its own also induced diabetogenic activity although to a lesser degree (Fig 5d). The dose of insulin for the insulin tolerance test was 0.08 units per kg live weight. Units for the insulin tolerance test are millimoles glucose/litre as a change over a one hour period. The calculation of diabetogenic activity is in effect a measure of an animal's responsiveness to insulin: after treatment with growth hormone or antipeptide antiserum the animal is less responsive to insulin. The degree to which it is less responsive is calculated on each animal by conducting an insulin tolerance test during a control period and one after treatment; the difference is essentially the diabetogenic activity.

Another effect of growth hormone in vivo is to alter fat metabolism. Subcutaneous fat (perirenal) was removed and assayed for lipogenic activity and lipid oxidation by  $^{14}\text{C}$  acetate incorporation into lipid and glycerol release respectively. Treatment of lambs with anti peptide immunoglobulin (to pGH 134-154), resulted in significant suppression of lipid synthesis. Similarly, administration of bGH on its own or in combination with antipeptide antiserum significantly decreased lipogenesis (measured as the amount of acetate (in micromoles) incorporated into triglyceride per gram of lipid in two hours). A similar profile of events was observed for lipid oxidation (Figure 5e). (Lipid oxidation is measured as the amount of acetate (in micromoles) converted to carbon dioxide per gram of lipid in two hours.)



Examples 20 to 41 Mapping of antigenic sites within the sequence region 112-159

Following the general procedure of Example 1, the following peptides, shown in Table 3, were prepared in order to map preferred antigenic sites within the sequence region 112-159 (n=5). The peptides were injected into sheep either without a carrier or with an ovalbumin carrier, as in Example 4, and the proportion of sheep responding were noted.

Table 3

(NC= no carrier. OVA=ovalbumin. nd= not done)

SEQUENCE	% RESPONDERS	
	NC	OVA
ALMRELEDGS	0	20
KLKDLEEGIQALMRELEDGS	0	80
RELEDGSPRAG	nd	nd
DLEEGIQALMRELEDGSPRAG	nd	nd
EDGSPRAGQIL	80	100
EGIQALMRELEDGSPRAGQIL	40	40
SPRAGQILKQT	0	60
QALMRELEDGSPRAGQILKQT	80	20
AGQILKQTYDK	40	60
MRELEDGSPRAGQILKQTYDK	80	0
ILKQTYDKFDT	40	80
LEDGSPRAGQILKQTYDKFDT	40	40
QTYDKFDTNLR	40	20
GSPRAGQILKQTYDKFDTNLR	40	20

DKFDTNLRSD	20	40
RAGQILKQTYDKFDTNLRSD	80	60
DTNLRSDALL	80	40
QILKQTYDKFDTNLRSDALL	80	0
LRSDALLKNY	40	80
KQTYDKFDTNLRSDALLKNY	40	100
DDALLKNY	40	60
YDKFDTNLRSDALLKNY	60	80

The positives range in binding of  $^{125}$ -labelled bGH from 200-2000 cpm over background at two different dilutions. Titres of individual sera vary between 1/50-1/5000) (in solid-phase RIA the level of responders always between 80-100%).

The results are shown graphically in Figure 1. The results indicate that the most antigenic regions lie between 133-159 and 128-147. The data also indicate that in cases the shorter peptides (about 10 amino acids) perform better than corresponding longer sequences of which the shorter sequence forms a part.

#### Examples 42 to 44

Peptide sequences pGH 134-154 and bGH 120-140 were prepared and injected into sheep or calves. Sheep or calf anti-growth hormone serum was thereby raised by the immunisation and titrated. Binding was determined in liquid phase RIA with either  $^{125}$ I-bGH or  $^{125}$ I-pGH. The results are shown in Table 4.

Table 4

Dilution of serum:	1/50	1/500	1/1000	1/5000
cpm/50 mcl of diluted serum assayed.				
$^{125}\text{I}$ -pGH (sheep)	1750	1050	865	265
$^{125}\text{I}$ -bGH (sheep)	2550	1890	995	375
$^{125}\text{I}$ -bGH (calf)	900	1097	925	505

The efficacy of the response did not depend on whether a carrier was employed. The same results were obtained whether the peptides were cross-linked to an ovalbumin carrier or polymerised to themselves.

Example 45

Pigs were immunised with fragments bGH 120-140 and pGH 134-154, each conjugated with ovalbumin, in complete Freund's adjuvant (250 mcg/pig) and challenged with the same dose in incomplete Freund's adjuvant after 28 days. Blood was taken after a further 10 days and assayed for antibody to bGH and pGH by solid phase ELISA. Essentially, plates were coated with bGH or pGH and binding of antiserum was determined with a peroxidase-conjugated rabbit immunoglobulin to swine Ig (Dako, Denmark) as described above (Examples 13 to 15) for the determination of sheep anti-hormone antibody in solid phase. Animals responded to both peptides, making antibodies which cross-reacted with the native hormones on the plates. Titres varied between 1/50 and 1/5000,

although responses were better for the peptide bGH/pGH 120-140. The results are shown in Table 5.

**Table 5**

Peptide	Response (%) - four animals per group	
	bGH	pGH
bGH 120-140	75	100
pGH 134-154	75	75

REFERENCES.

Aston, R., Holder, A.T., Preece, M.A. and Ivanyi, J.  
(1986) J. Endocrinol. 110, 381-388.

Aston, R., Holder, A.T., Ivanyi, J. and Bomford, R.  
(1987) Molec. Immunol. 24, 143-150.

Holder, A.T. Aston, R. Preece, M.A. and Ivanyi, J.  
(1985) J. Endocrinol. 107, R9-R12.

Cole, H.H., Dewey, R., Geschwind, I.I. and Chapman, M.  
(1975) Biol. Reprod 12, 516

Ferguson, K.A. (1954) Nature 174, 411

Ng, F., Henderson, T., Bromley, J. and Bornstein, J  
(1983) 30, 63-71.

Pullin, C. O., Harcourt, J.A., Ng, F.M. and Bornstein, J.  
(1981) Int. J. Peptide Protein Res. 18, 318-323.

- Smith, R. W. and Walsh, A. (1984) Effects of lactation on the metabolism of sheep adipose tissue. Research in Veterinary Science Vol. 37.
- Smith R.W. and Walsh, A. (1988) Effects of Pregnancy and lactation on the Metabolism of bovine adipose tissue. Research in Veterinary Science. Vol. 44.
- Yang, Y. T. and Baldwin, R.L. (1973) J. Dairy Science. 44
- Isaksson, O.G.P., Edan, S. Jansson, J-O (1985) Ann. Rev. Physiol. 47, 483-499.
- Nicoll, C.S., Mayer, G.L., and Russell, S.M. (1986) Endocrine Rev. 7, 169-203.
- Rolands, I.W. (1939) J. Endocrinol. 1, 177
- Shechter, Y., Chang, K.J., Jacobs, S. and Cuatrecasas, P. (1979) Proc. Natl. Acad. Sci. USA. 76, 2720
- Shechter, Y., Hernaez, L., Schlessinger, J and Cuatrecasas, P. (1979) Nature 278, 835-838.
- Thompson, K.W. Proc. Soc. Expl. Biol. NY 35, 640.
- Goodfriend, T.L., Webster, M.E., McGuire, J.S. (1970) J. Clin. Endocrinol. Metab. 30, 565-572.
- Aston, R., Cooper, L., Holder, A.T. Ivanyi, J. and Preece, M.A. (1985) Molec. Immunol. 22, 271-275.

CLAIMS

1. An antigenic molecule which causes antibodies to be raised against at least some of the 112 to 159 region of a natural growth hormone.
2. A molecule (other than a natural growth hormone) at least part of which is antigenically equivalent to an oligopeptide selected from residues 112 to 159 of a natural growth hormone.
3. A molecule as claimed in claim 1 or 2, wherein the growth hormone is bovine, ovine, porcine or chicken growth hormone.
4. A molecule as claimed in claim 1 or 2 wherein the oligopeptide is of from six to twenty amino acid residues.
5. A molecule as claimed in claim 1 or 2, wherein the molecule or part of the molecule which is antigenically equivalent to the oligopeptide comprises a sequence of amino acid residues which is identical to or conformationally similar to the oligopeptide.
6. A molecule as claimed in claim 1 or 2 comprising the amino acid sequence:

AGQILKQTYDKFDTNLRSDDA

or

IQALMRELEDGSPRAGQILKQ

or

EDGSPRAGQIL

34

or

AGQILKQTYDK

or

RAGQILKQTYDKFDTNLRSD

or

DTNLRSDALL

or

KQTYDKFDTNLRSDALLKNY

or

LRSDALLKNY

or an active fragment thereof and/or conservative mutant thereof or salts thereof.

7. A method of promoting the activity of growth hormone (or a substance having growth hormone activity) comprising administering to a vertebrate an effective amount of a molecule as claimed in claim 1 or 2.

8. A method as claimed in claim 7, comprising simultaneously or subsequently administering growth hormone or a substance having growth hormone activity.

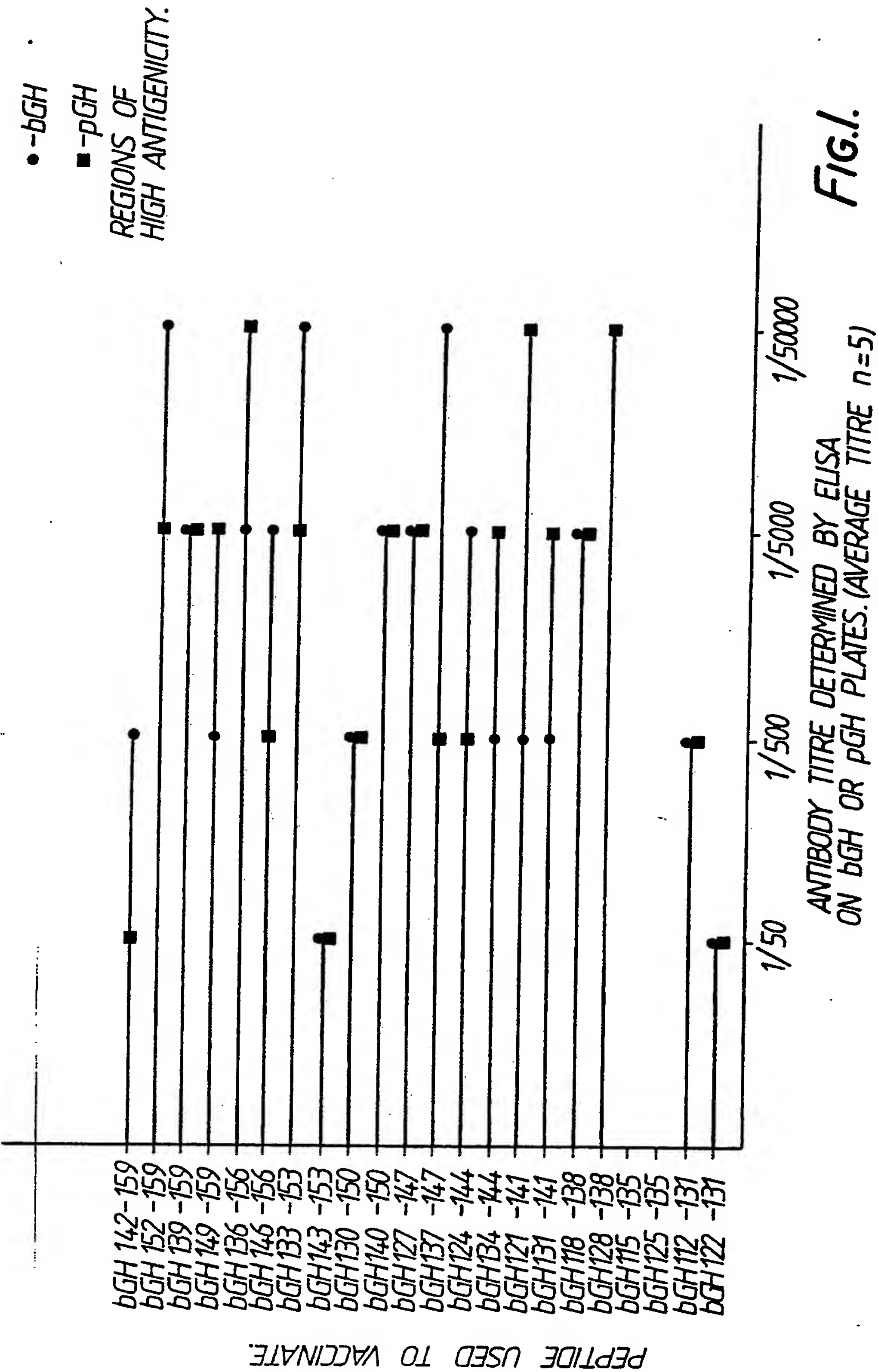
9. The use of a molecule as claimed in claim 1 or 2 in the preparation of an agent for use in the promotion of the activity of growth hormone or a substance having growth hormone activity.

10. The use as claimed in claim 8 in conjunction with the use of growth hormone or a substance having growth hormone activity in the preparation of an agent for simultaneous or subsequent administration.



11. A molecule as claimed in claim 1 or 2 comprising a carrier peptide or protein.
12. A molecule as claimed in claim 2 which has a plurality of copies of the said antigenically equivalent part.
13. A pharmaceutical or veterinary composition comprising a molecule as claimed in claim 1 or 2 in conjunction with a pharmaceutically or veterinarily acceptable carrier.
14. A composition as claimed in claim 13 comprising a substance having growth hormone activity.
15. An antibody to a molecule as claimed in claim 1 or 2.
16. A pharmaceutical or veterinary composition comprising an antibody as claimed in claim 15 in conjunction with a pharmaceutically or veterinarily acceptable carrier.

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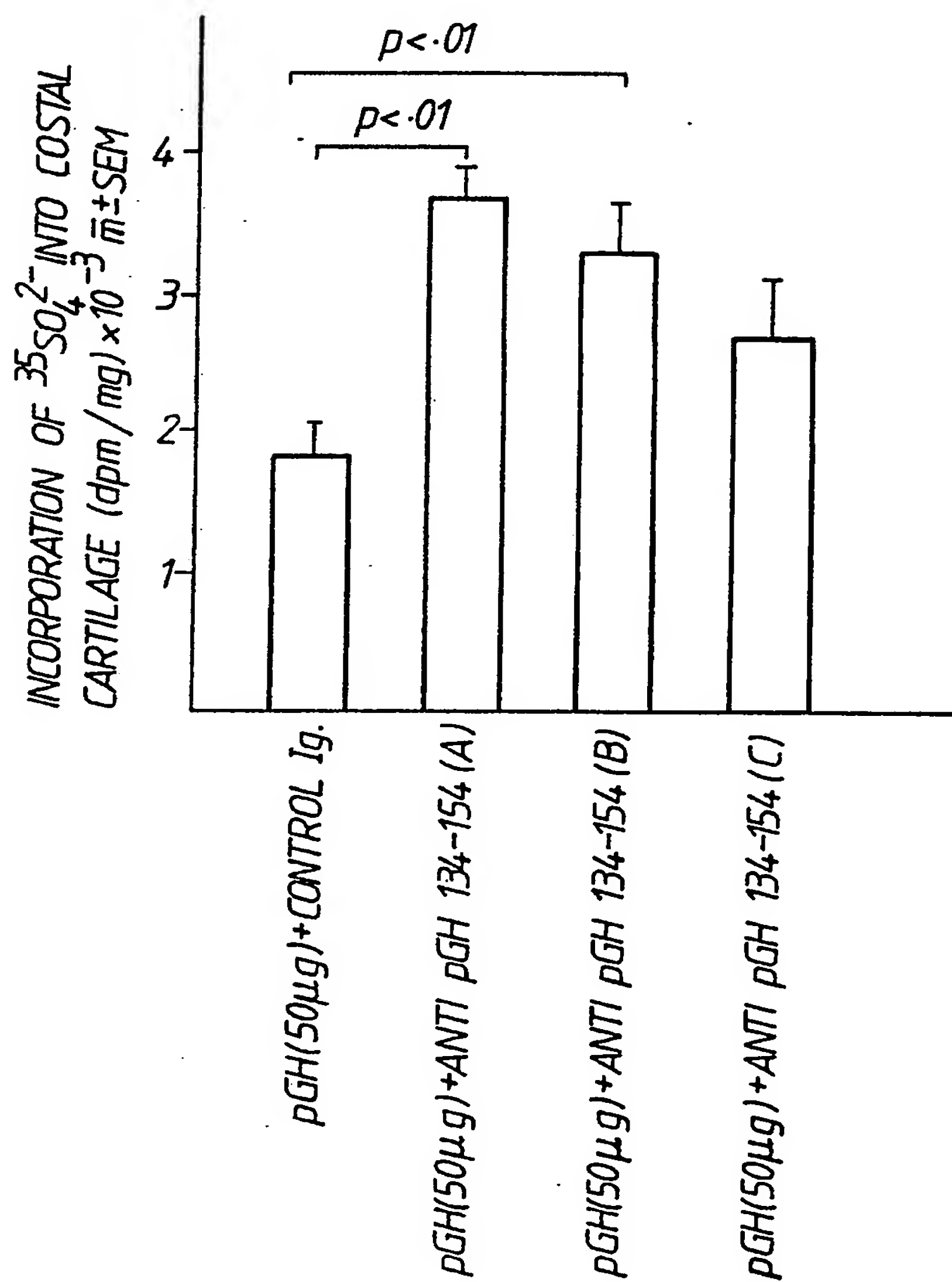


FIG. 2.

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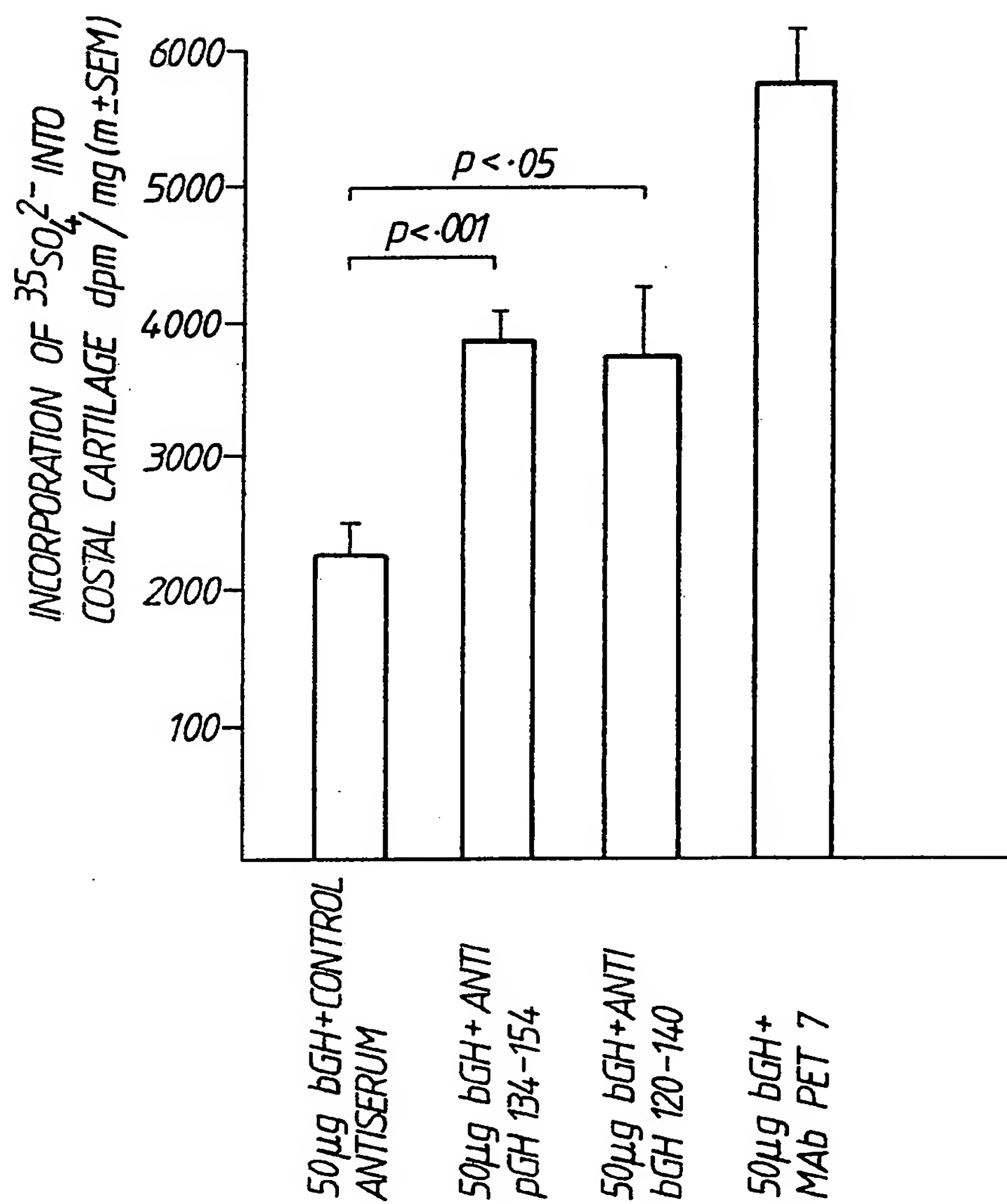


FIG.3

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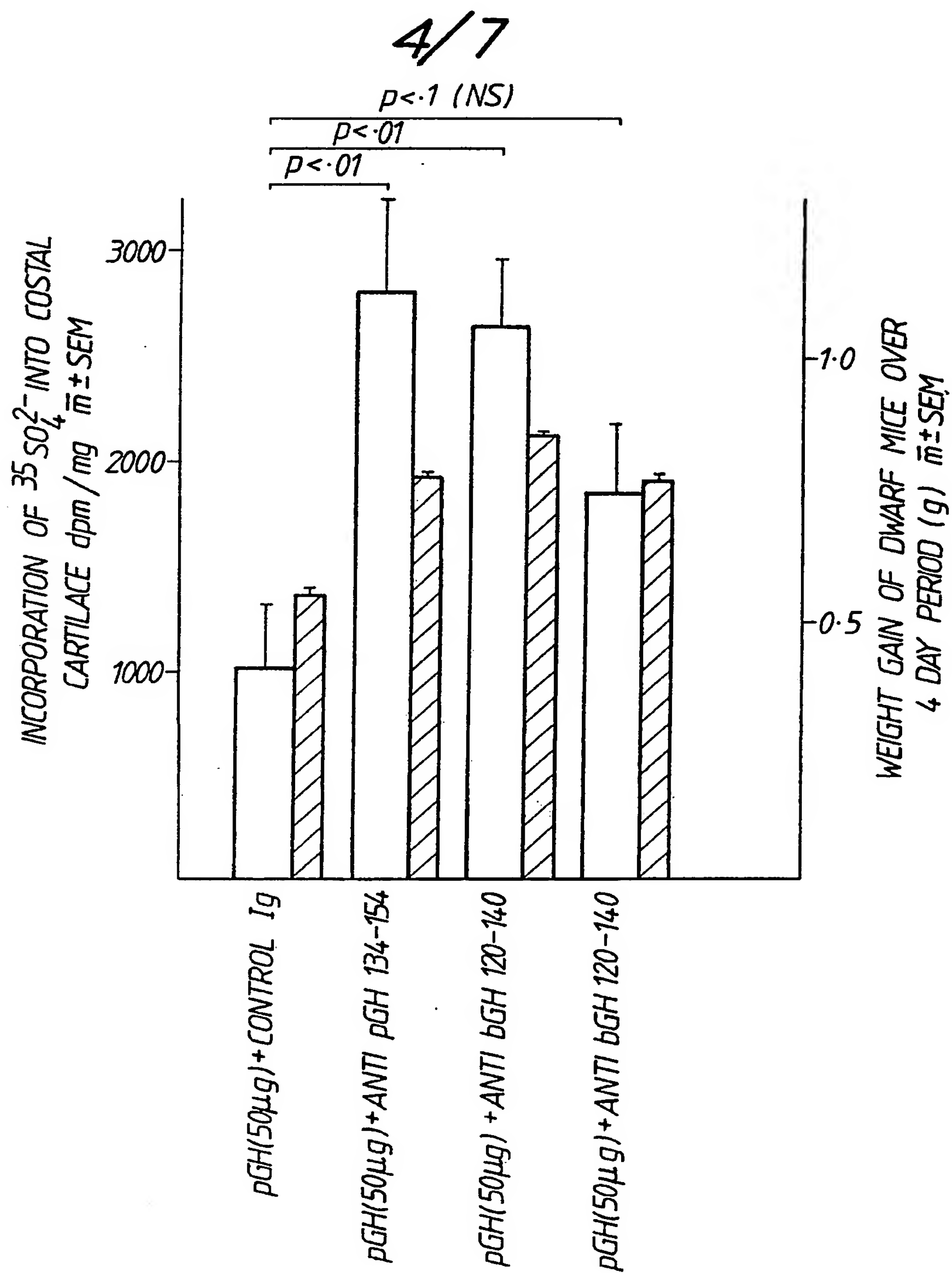
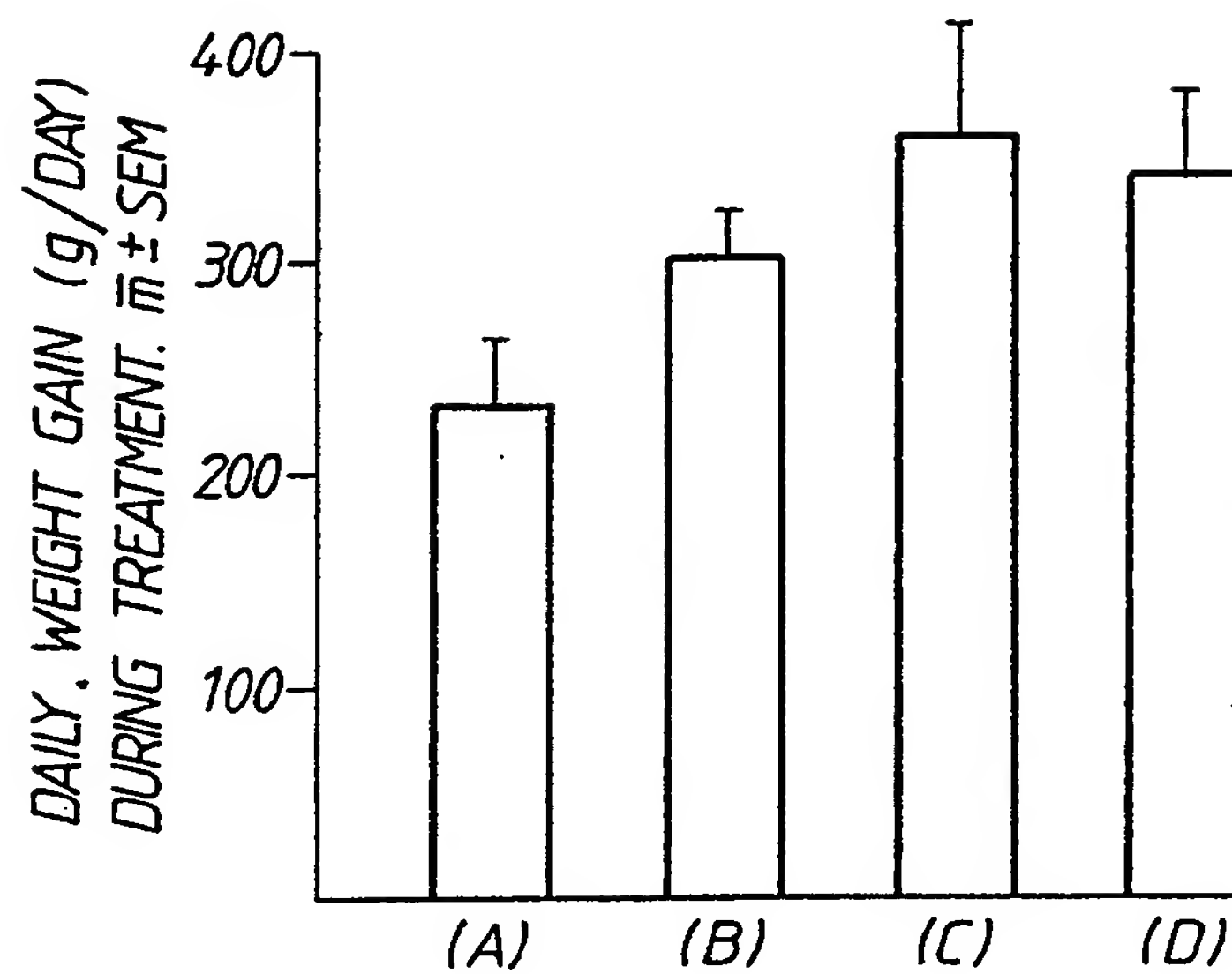


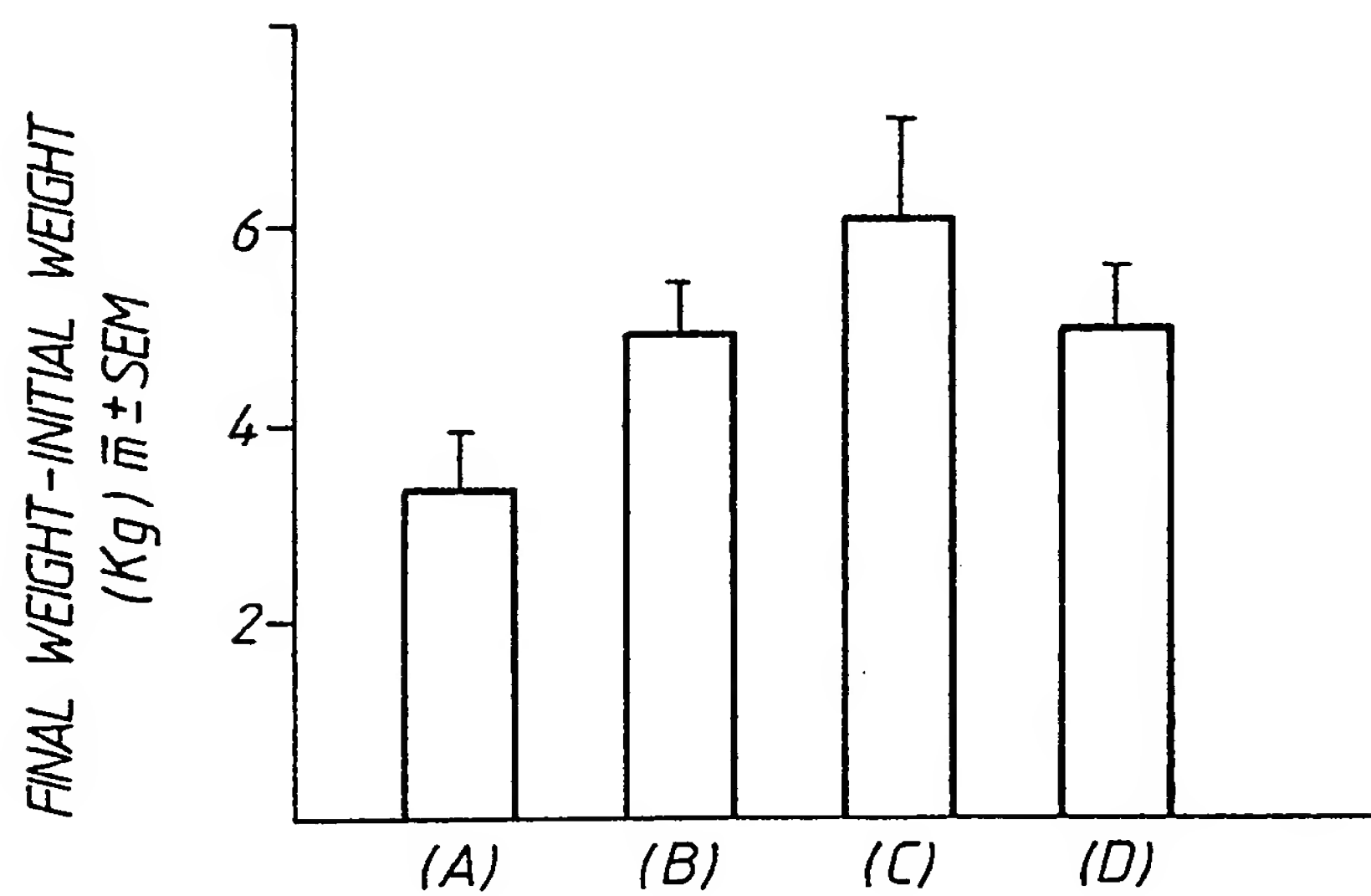
FIG. 4.

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(a)

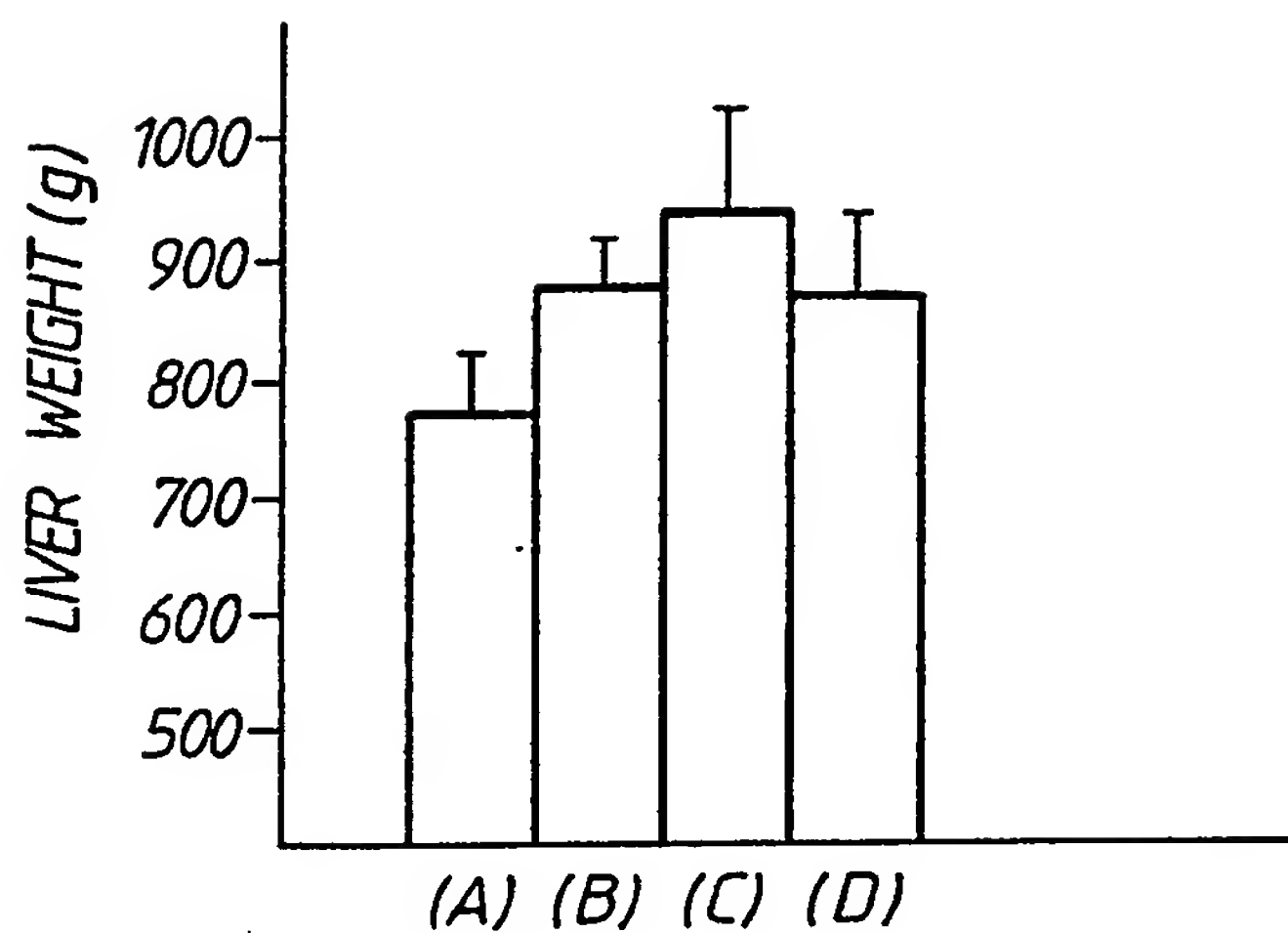


(b)

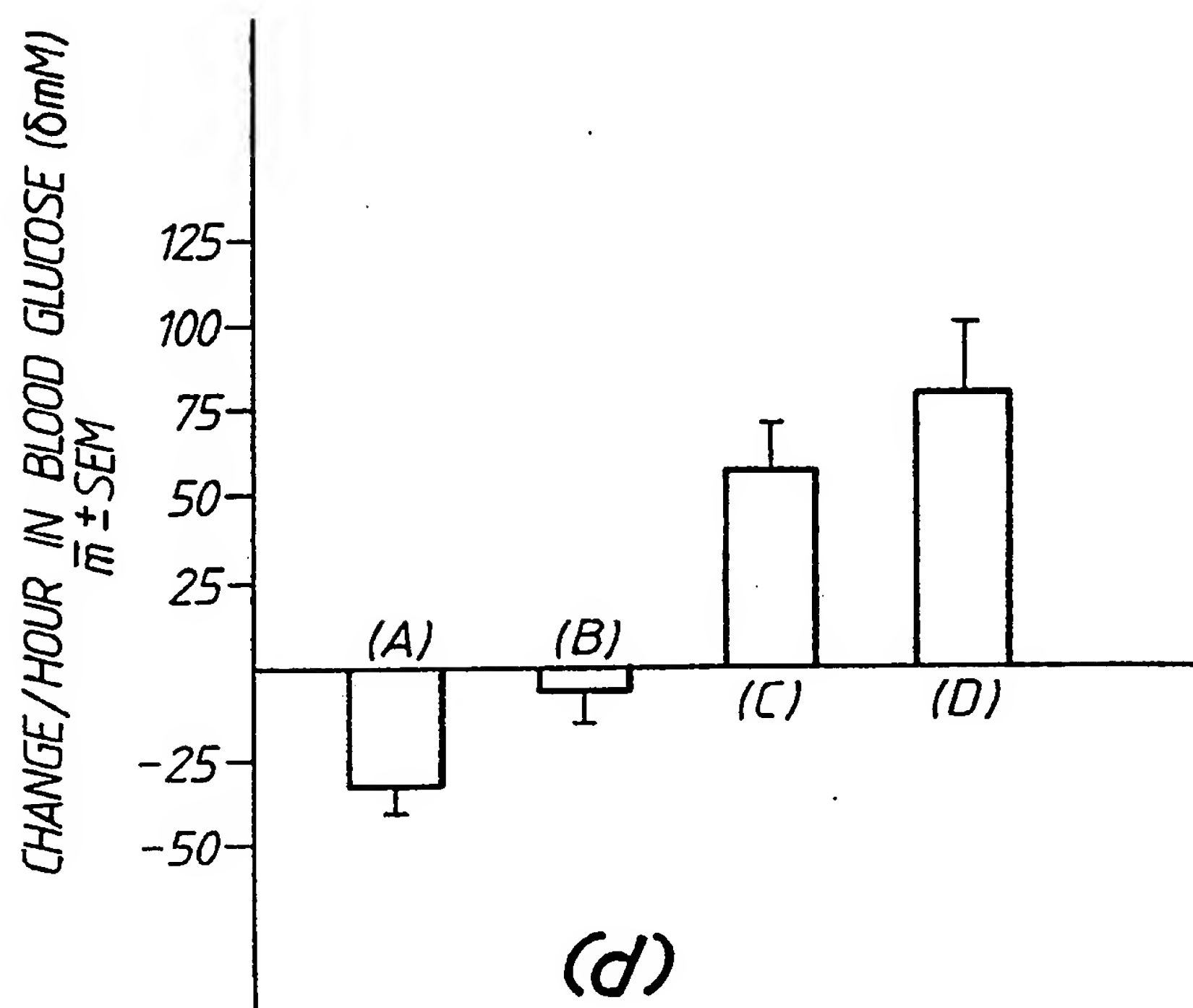
FIG. 5.

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(c)



(d)

Fig.5.

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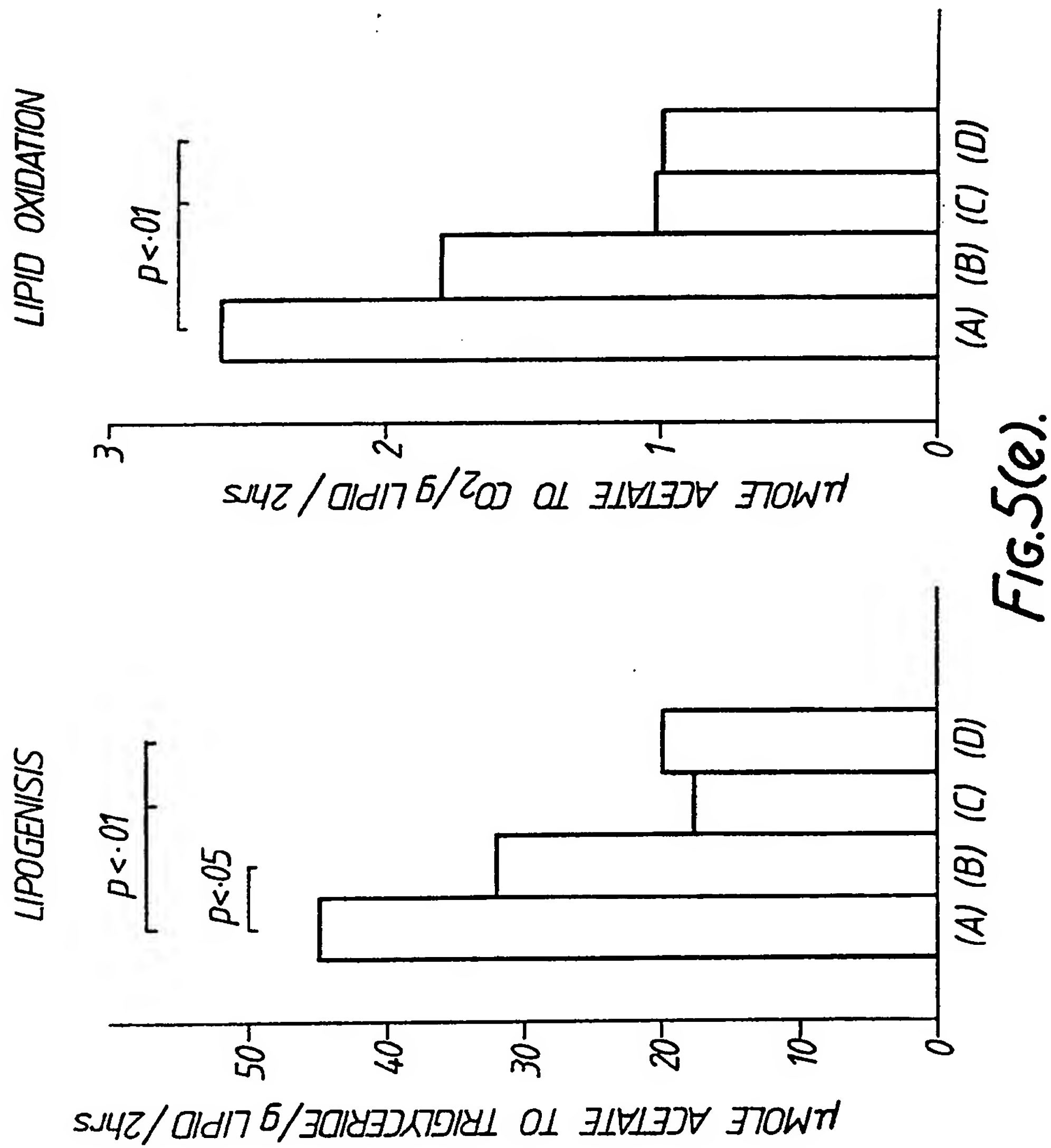


FIG.5(e).



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>4</sup> :</b> <b>C 07 K 7/06, 7/10; // A 61 K 37/36</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 89/ 00166</b> <b>(43) International Publication Date:</b> 12 January 1989 (12.01.89)
<b>(21) International Application Number:</b> PCT/GB88/00528 <b>(22) International Filing Date:</b> 6 July 1988 (06.07.88) <b>(31) Priority Application Numbers:</b> 8715858 8715859 8715866 <b>(32) Priority Dates:</b> 6 July 1987 (06.07.87) 6 July 1987 (06.07.87) 6 July 1987 (06.07.87) <b>(33) Priority Country:</b> GB <b>(71) Applicant (for all designated States except US):</b> PEP- TIDE TECHNOLOGY LTD [AU/AU]; 4-10 Inman Road, P.O. Box 444, Dee Why, NSW 2099 (AU). <b>(71)(72) Applicant and Inventor:</b> ASTON, Roger [GB/AU]; Lot 2340 Highs Road, West Pennant Hills, Sydney, NSW 2120 (AU).		<b>(74) Agents:</b> SHEARD, Andrew, Gregory et al.; Kilburn & Strode, 30 John Street, London WC1N 2DD (GB). <b>(81) Designated States:</b> AT (European patent), AU, BE (Eu- ropean patent), BR, CH (European patent), DE (Eu- ropean patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent), US. <b>Published</b> <i>With international search report</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i> <b>(88) Date of publication of the international search report:</b> 26 January 1989 (26.01.89)
<b>(54) Title:</b> GROWTH HORMONE RELATED PEPTIDE  <b>(57) Abstract</b>  Antigenic molecules cause antibodies to be raised against at least some of the 112 to 159 region of a natural growth hormone when injected <i>in vivo</i> . The molecules can comprise portions of the 112 to 159 region. The antibodies are believed to bind to growth hormone on administration and enhance its effect.		


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# INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 88/00528

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC <sup>4</sup> : C 07 K 7/06; C 07 K 7/10; //A 61 K 37/36		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
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IPC <sup>4</sup>	C 07 K 7/00; A 61 K 37/00; A 61 K 39/00; C 12 P 21/00	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b>		
Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	Chemical Abstracts, vol. 89, 1978, (Columbus, Ohio, US) F. Chillemi et al.: "Synthesis of fragments with amino acid sequences of growth hormones" see page 70, abstract no. 71230y & Excerpta Med. Int. Congr. Ser. 1976, 381 (Growth Horm. Relat. Pept.) 50-63 --	1
X	Chemical Abstracts, vol. 80, 1974, (Columbus, Ohio, US) F. Chillemi: "Solid-phase synthesis of peptides with growth-promoting activity" see page 452, abstract no. 108849z & Gazz. Chim. Ital. 1973, 103(6-7) 657-67 --	1
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
24th November 1988	20 DEC 1988	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	 <b>P.C.G. VAN DER PUTTEN</b>	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Chemical Abstracts, vol. 99, 1982, (Columbus, Ohio, US) T.K. Surowy et al.: "Immunological and receptor-binding properties of frag- ments resulting from cyanogen bromide cleavage of bovine growth hormone" see page 71, abstract no. 152330q & J. Protein Chem. 1983, 2(3), 195-208 --	1
X	Endocrine Reviews, vol. 7, no. 2, 1986 The Endocrine Soc. (US) C.S. Nicoll et al. "Structural features of prolactins and growth hormones that can be related to their biological properties" pages 169-203, see the whole document (cited in the application)	1
Y	--	2-16
X	Endocrine Reviews, vol. 4, no. 2, 1983 The Endocrine Soc. (US) W.L. Miller et al.: "Structure and evolution of the growth hormone gene family" pages 97-130, see the whole document	1
Y	--	2-16
Y	EP, A, 0044710 (SCRIPPS CLINIC AND RESEARCH FOUNDATION) 27 January 1982, see the whole document	1-16
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**FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET**

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

**This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:**

1. ☐ Claim numbers ....., because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim numbers ....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

**This International Searching Authority found multiple inventions in this International application as follows:**

Please refer to Form PCT/ISA/206 dated October 14th, 1988.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

**Remark on Protest**

- ☒ The additional search fees were accompanied by applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

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EP-A- 0044710	27-01-82	AU-A- 7283681	21-01-82
		JP-A- 57118545	23-07-82
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		AU-B- 559234	05-03-87
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